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PCR-based identification of *Pythium* spp. causing cavity spot in carrots and sensitive detection in soil samples

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On the basis of ITS sequences PCR primers were designed for the identification of the five *Pythium* species found to be most important for the development of carrot cavity spot in Norway: *P. intermedium, P. sulcatum, P. sylvaticum, P. violae* and *P.* '*vipa*'. The *P.* '*vipa*' isolates had a unique ITS sequence, differed morphologically from all other *Pythium* isolates, and thus probably represent a new species. The PCR primers were species-specific with no cross-reaction to other *Pythium* species or to fungal isolates from carrot tested. The detection limits varied for the different primer pairs. The two most sensitive assays allowed detection of as little as 5 fg DNA. All five *Pythium* species could be detected in lesions from diseased carrots. Weak positive signals were obtained from some carrot samples without symptoms. PCR assays allowed detection of pathogens in soil. In samples of soil known to produce cavity spots on cropped carrots, strong signals were obtained. In several soil samples more than one of the five *Pythium* species could be detected. The utilization of this diagnostic PCR assay in analysis of field soil and carrot tissue might in the future be exploited to reduce the incidence of this serious carrot disease.

Keywords: *Daucus carota*, internal transcribed spacer, molecular diagnosis, ribosomal DNA, soilborne disease

Introduction

Cavity spot is one of the most economically important diseases of carrot (*Daucus carota*). The disease was first described in the USA in 1961 (Guba *et al*., 1961), but has since been found in a number of countries including Israel, Egypt, Canada, Australia and several European countries, as reviewed by Hiltunen & White (2002). In Norway the disease was well established by 1980 (Årsvoll, 1980) and was shown to affect up to 50% of the carrot crop (Hermansen, 1992). Cavity spot develops during the growing season and at harvest the symptoms can be seen as small, sunken lens-shaped cavities. If environmental conditions are favourable, the size of the cavities may increase rapidly and cavities up to 1 cm in diameter can be found.

The fact that multiple *Pythium* species were the cause of cavity spot in carrots was not known until the 1980s (Lyshol *et al*., 1984; Groom & Perry, 1985; White, 1986). Since then several surveys have been performed to identify the different *Pythium* species involved and their relative importance in different geographic areas. In most countries the slow-growing species *P. sulcatum* and *P. violae* are considered the most important, but other species are reported to contribute to the development of disease (Hiltunen & White, 2002; Allain-Boulé *et al*., 2004a,b; Suffert & Guibert, 2007). Five species of *Pythium* are the most important in causing cavity spot in Norway (Hermansen *et al*., 2007). These include *P. violae*, *P. sulcatum*, *P. sylvaticum*, *P. intermedium* and what is believed to be a new species, given the preliminary name *P.* '*vipa*'.

Development of diagnostic methods enabling the detection of cavity-spot-causing species in soil could be of great importance in limiting economic losses caused by cavity spot. Such a test could potentially be used to analyse fields in order to avoid cropping carrots where the corresponding *Pythium* species are found and the consequent risk of developing cavity spot is high.

Various methods have been employed to detect species causing cavity spot. Polyclonal as well as monoclonal antibodies have been developed for the detection of *P. sulcatum* and *P. violae* (Lyons & White, 1992; White *et al*., 1994; Kageyama *et al*., 2002). The antibodies showed moderate to considerable cross-reaction to other *Pythium* species that are not pathogenic to carrots. An indirect competitive ELISA assay allowed detection of *P. sulcatum* and *P. violae* in soil and carrot tissue (White *et al*., 1996), but did not correlate well with the development

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of the disease in Norway (Hermansen & Dragland, 2000), probably because the ELISA did not target all *Pythium* species in the cavity spot disease complex. Several DNA-based methods were developed for diagnosis of *Pythium* species, e.g. PCR-RFLP, reverse dot blot hybridization, DNA macroarrays and AFLP fingerprinting (Wang & White, 1997; Lévesque *et al*., 1998; Garzón *et al*., 2005; Lievens *et al*., 2005), but none of these addressed the diversity of species causing cavity spot. Wang & White (1996) described the development of a *P. violae*-specific PCR primer and Wang *et al*. (2003) developed species-specific PCR assays for several *Pythium* species, including *P. sulcatum*. However, the sensitivity of these tests and their usefulness for detecting pathogenic *Pythium* species in soil or carrot tissue has not been described.

The objectives of this study were (i) to develop PCR assays for the species-specific identification of the cavity-spot-causing *Pythium* species most frequently found in Norway, including *P. sulcatum*, *P. violae*, *P. sylvaticum*, *P. intermedium* and *P.* '*vipa*', and (ii) to investigate the sensitivities of these tests to see if they potentially could be used to detect the organisms in soil and carrot tissue. The ultimate goal is to relate the quantitative detection of cavity-spot-causing *Pythium* species in soil intended for cropping in carrot with cavity spot disease incidence.

Materials and methods

Oomycete and fungal isolates

Isolates from the culture collection at Bioforsk, Plant Health and Plant Protection Division, Norway, were used in this study. All strains used were of Norwegian origin. Strains of *P. intermedium, P. sulcatum, P. sylvaticum, P. violae* and *P.* '*vipa*' were isolated from carrots with cavity spot symptoms (Table 1; Hermansen *et al*., 2007). The carrots with cavity spots were collected from farms located in the eight counties Rogaland, Aust Agder, Vest Agder, Telemark, Vestfold, Østfold, Hedmark and Nord Trøndelag. These counties are all in the main carrot-growing areas of Norway, i.e. the south, east and middle parts of the country. A number of other *Pythium* species and several additional fungal isolates originating from carrots were identified morphologically and included in the primer-specificity test (Table 2). None of these other *Pythium* species has been found to cause disease in carrots in Norway, but *P. aphanidermatum*, *P. coloratum*, *P. irregulare*, *P. rostratum* and *P. ultimum* have been described as carrot pathogens in other countries. The additional *Phytophthora* isolates and fungal isolates were either included in the analysis because they were known to be carrot pathogens, or because they are frequently present in soil where carrots are usually grown (Table 2).

Table 1 *Pythium* species, isolate code and amplification result for the PCR assays developed in this study. PCR utilized primer pairs (A) PINTERf/r, (B) PSULCf/r, (C) PSYLf/r, (D) PVIOLf/r and (E) P*VIPA*f/r as described in this study. Successful (+) and unsuccessful (–) amplification was determined based on production of a PCR product of the correct size

Pythium species	Isolate	ITS region similar to accession no.	PCR amplification				
			A	B	C	D	E
P. intermedium	97-84·II	DQ528742	+	–	–	–	–
P. intermedium	96-137	DQ528742	+	–	–	–	–
P. intermedium	96-206	DQ528742	+	–	–	–	–
P. sulcatum	96-03	DQ528744	–	+	–	–	–
P. sulcatum	96-29	DQ528744	–	+	–	–	–
P. sulcatum	96-127	DQ528744	–	+	–	–	–
P. sulcatum	96-133	DQ528744	–	+	–	–	–
P. sulcatum	96-141	DQ528744	–	+	–	–	–
P. sulcatum	96-237	DQ528744	–	+	–	–	–
P. sulcatum	96-234	DQ528745	–	+	–	–	–
P. sulcatum	96-235	DQ528745	–	+	–	–	–
P. sulcatum	97-16·I	DQ528745	–	+	–	–	–
P. sulcatum	97-22·I	DQ528745	–	+	–	–	–
P. sylvaticum	M33	DQ528741	–	–	+	–	–
P. sylvaticum	M71	DQ528741	–	–	+	–	–
P. sylvaticum	96-183	DQ528741	–	–	+	–	–
P. sylvaticum	96-185	DQ528741	–	–	+	–	–
P. sylvaticum	98-113	DQ528741	–	–	+	–	–
P. sylvaticum	96-66·2	DQ528741	–	–	+	–	–
P. sylvaticum	99-20A	DQ528741	–	–	+	–	–
P. sylvaticum	96-05	DQ528741	–	–	+	–	–
P. violae	98-44	DQ528740	–	–	–	+	–
P. violae	96-142	DQ528740	–	–	–	+	–
P. violae	96-121	DQ528740	–	–	–	+	–
P. violae	96-91A	DQ528740	–	–	–	+	–
P. violae	96-79	DQ528740	–	–	–	+	–
P. '*vipa*'	96-224	DQ528743	–	–	–	–	+
P. '*vipa*'	96-223	DQ528743	–	–	–	–	+

Table 2 Species and isolate codes of fungal isolates originating from carrots, and of other oomycetes from the culture collection at Bioforsk, tested with the *Pythium* PCR assays developed in this study

Pathogen species	Strain no.
Botrytis sp.*	1075
Botrytis sp.*	358/3
Cylindrocarpon sp.*	3292
*Fibularhizoctonia carotae**	841/1
*F. carotae**	663/2
*F. carotae**	3414A
Fusarium sp.*	3137/5
*Mycocentrospora acerina**	3194
*M. acerina**	56/1
*M. acerina**	3227
*M. acerina**	859/1
Phytophthora cryptogea	3081/3
Phytophthora infestans	T96-665
Pythium angustatum	M64
Pythium aphanidermatum[a]	1080
Pythium aquatile	972/1
Pythium coloratum[a]	M80
Pythium connatum	M101
Pythium deliense	M94
Pythium dissotocum	97-48·II
Pythium irregulare[a]	M75
Pythium mamillatum	M74
Pythium middletonii	M42
Pythium monospermum	M68
Pythium myriotylum	M85
Pythium rostratum[a]	3083/4
Pythium tracheiphilum	A98-47/3
Pythium torulosum	M105
Pythium ultimum[a]	3089
Pythium group F	M83
Pythium group T	3047
Pythium group HS	M61
*Rhizoctonia solani**	3103
Rhizoctonia sp.	941/90
Stemphyllium sp.	3172
Verticillium sp.	2056

*Known pathogen of carrots in Norway.
[a]Known to cause disease in carrots in other counties, but not known to do so in Norway.

DNA extraction

Oomycete and fungal isolates were grown on potato dextrose agar covered with cellophane at 20°C for 4–6 days. The mycelium was harvested and ground to a fine powder in liquid nitrogen and DNA extracted using the DNeasy Plant Mini Kit (QIAGEN Inc.) according to the manufacturer's instructions.

For the extraction of DNA from soil, 0·25 g of a representative soil was homogenized and the cells disrupted by adding one 3-mm tungsten carbide bead (QIAGEN Inc.) to the tube and using the FastPrep Instrument, FP120, (Bio101 Savant) for 30 s at speed 5·5. DNA extraction was performed using the PowerSoil DNA Isolation Kit from Mo Bio Laboratories, Inc.

Carrots with and without visible symptoms of cavity spots were collected at harvest from fields with commercial carrot production. To extract DNA, roots were washed in water and peels were taken from the top and tip of each carrot. This tissue was freeze-dried overnight and the dried samples ground to a fine powder with a mortar and pestle. About 50 mg dried carrot tissue powder were transferred to a microcentrifuge tube and DNA extracted using the GenElute Plant Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's recommendations, except that DNA was eluted from the binding column once with 100 μL TE buffer, pH 7·5, pre-warmed to 65°C.

After initial extraction, DNA samples from carrots and soil were further purified using Micro Bio Spin Chromatography columns (BioRad Laboratories Ltd) filled with insoluble polyvinylpolypyrrolidone (PVPP). Each column was loaded with 400 μL sterile water, placed in a microcentrifuge tube and centrifuged for 5 min at 1500 ***g***. The column was transferred to a new microcentrifuge tube, and DNA was loaded onto the PVPP surface. The purified DNA was collected in the bottom tube by centrifugation at 1500 ***g*** for 5 min.

Sequencing of rDNA regions

DNA extracted from 28 strains, representing five *Pythium* species isolated from cavity spot lesions (Table 1; Hermansen *et al*., 2007) was amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). Amplification of the ITS (internal transcribed spacer) region of rDNA was performed using an initial denaturation at 94°C for 2 min followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN Inc.) and sequenced using an ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Both strands of the entire ITS region were sequenced using the primers ITS1, ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (White *et al*., 1990).

DNA analysis and design of species-specific primers

DNA sequences were analysed using the SEQMAN™II 4·03 program (Lasergene Sequence Analysis Software, DNASTAR Inc.). The different ITS genotypes were submitted to GenBank and the corresponding accession numbers are listed in Table 1. The BLAST network services at the National Centre for Biotechnology Information (Altschul *et al*., 1997) were used to search for sequence similarity to previously sequenced rDNA regions.

The ITS sequences of *P. intermedium, P. sulcatum, P. sylvaticum, P. violae* and *P.* '*vipa*' were aligned using the MegAlign Multiple Sequence Alignment program (Lasergene Sequence Analysis Software, DNASTAR Inc.) and compared to those of other *Pythium* species to which

Table 3 Sequence of the species-specific PCR primers, annealing temperatures and expected size of amplicon for each primer pair used to detect *Pythium* species possibly causing carrot cavity spot

Target species	Size[a]	Annealing temperature	Primer name	Primer sequences (5′→3′)[b]
P. intermedium	380 bp	60°C	PINTERf/PINTERr	ATGCAGAGGCTGAACGAA/CTGTATTCATAGCCGAAACGA
P. sulcatum	646 bp	60°C	PSULCf/PSULCr	GCCGCTTTATTGTGGTCT/TCTTCTTTACCCCACAAGTGA
P. sylvaticum	330 bp	56°C	PSYLf/PSYLr	CGCTGTGGTTGGTATATTTGT/GCCAATTGCACAAGTACAAA
P. violae	352 bp	61°C	PVIOLf/PVIOLr	ATGTGTGTGTGCGGGACT/CCACTCCCCAAAGAGAGAAGT
P. '*vipa*'	329 bp	57°C	P*VIPA*f/P*VIPA*r	CAGCGGTTGGTATATTCGTT/AAAAAGAAGTGCACAAATAGATGA

[a]Expected size of PCR product.
[b]The PCR primers are covered by patent application PCT/GB03/04712 owned by Carrotech AS.

they were most similar. Primers were designed manually to the most variable sequence regions. Primer sequences are listed in Table 3. A phylogenetic tree was calculated from the ClustalW Multiple Sequence Alignment using the MegAlign Multiple Sequence Alignment program.

Polymerase chain reaction

PCR reactions were performed in a total volume of 25 μL with final concentrations of 50 mM KCl, 10 mM Tris-HCl (pH 8·3), 0·2 mM dNTPs, 0·1 mg mL^{-1} bovine serum albumin and 1·5 mM $MgCl_2$ in all assays, except for primer pair P*VIPA*f/r, when the $MgCl_2$ concentration was 3 mM. For each reaction 25 pmol of each primer and 0·6U AmpliTaq polymerase (Applied Biosystems) were used. The primer-specificity tests were performed with approximately 5 ng DNA. When testing carrot tissue or soil samples for the presence of the cavity spot pathogens, 1 μL of the extracted DNA was used as template. A PCR reaction using universal primers ITS3 and ITS4 (White *et al.*, 1990) was used as a positive control for DNA extracted from both carrot tissues and soil samples in order to evaluate DNA quality and reveal the possible presence of PCR inhibitors. Amplifications were performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) programmed for initial denaturation at 94°C for 5 min followed by 45 cycles of 20 s at 94°C, 30 s annealing and 30 s at 72°C. For *P. sylvaticum* and *P.* '*vipa*' the annealing temperature was 56 and 57°C, respectively. For *P. sulcatum* and *P. intermedium* the annealing temperature was 60°C and for *P. violae* the annealing temperature was 61°C. Ten microlitres of the PCR products were separated by electrophoresis through 1·2% agarose gels, stained with EtBr and photographed in UV light on a GelDoc 1000 (BioRad Laboratories Ltd).

Sampling of soil

From the fields to be tested for cavity spot pathogens, 10 subsamples of soil were collected from the upper 20 cm of the soil for every 500 m^2. The subsamples were bulked together, mixed and 250 mL of this sample were stored at 4°C until analysis. Before analysis this soil sample was further thoroughly mixed and 0·25 g used for DNA extraction as described above. To test the sensitivity of the PCR assays in soil, 12 random fields that should be used for carrot production were tested in spring. In another experiment eight samples of soil were collected from a field with a previous history of cavity spot. Of these samples four were collected at harvest and four were collected 3 weeks before harvest.

Sensitivity tests

Sensitivity of the PCR assays was evaluated using tenfold dilutions of genomic DNA ranging from 50 ng to 5 fg from *P. intermedium*, *P. sulcatum*, *P. sylvaticum*, *P. violae* and *P.* '*vipa*'. Two independent DNA preparations from each species were analysed twice using the primer pairs PINTERf/r, PSULCf/r, PSYLf/r, PVIOLf/r and P*VIPA*f/r.

Test of primer specificity in soil samples

Each of the five different primer pairs were tested for specific amplification of *Pythium* target DNA in samples of total DNA extracted from soil. From five different carrot fields, DNA samples resulting in clear amplified fragments with the same molecular weight as the positive control were selected for further analysis. The PCR products were purified and the DNA sequence determined as described above, using one of the specific primers as the sequencing primer. One of the five originally selected PCR products was randomly chosen and cloned into *Escherichia coli* using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. From each isolate five clones were selected for PCR amplification using the primers PUC1 (5′-TATCTGCGCCTCTGCTGAA-3′) and PUC2 (5′-CCAAATACTGTTCTTCTAGTG-3′) and the DNA sequence of the amplified insert of each of the five clones was determined.

Characterization of PCR products from soil samples

The DNA quantities in the PCR products resulting from the analysis of soil samples were determined as previously described (Halstensen *et al.*, 2006). The pixel volumes of the bands (area of the band on the gel multiplied by the intensity) were determined using Quantity One 1-D Analysis Software, version 4·5·0 (Bio-Rad). The negative water control with no *Pythium* DNA was set to a quantity

of zero and the quantity of the PCR fragment resulting from 50 ng of the *Pythium* strain used as the positive control was regarded as 100%. The software calculated the unknown samples as percentages of the positive control sample. All PCR reactions were run twice.

Results

ITS sequence and morphological characterization

The ITS sequences of all five *P. violae* isolates were identical. This sequence did not differ from that of *P. violae* strain CBS 178·86, also isolated from carrot (GenBank Accession No. AY598715), but was significantly different from that of *P. violae* CBS 132·37 isolated from *Viola* (GenBank Accession No. AY598717; Fig. 1). Two genotypes were found among the 10 isolates of *P. sulcatum* differing in only two nucleotides. These two genotypes could also be distinguished morphologically. One group did not produce zoospores and had one or two antheridia per oogonium. The other group readily produced zoospores and most often had two or three antheridia per oogonium, and was thus more similar to the type strain described by van der Plaats-Niterink (1981). Only one ITS genotype was found in the three isolates of *P. intermedium* and eight isolates of *P. sylvaticum* and these were identical to ITS sequences already present in the GenBank. No sequence in GenBank was found to be identical to the ITS sequence of the two strains of *P.* '*vipa*'; the most similar sequence was the ITS sequence of *P. irregulare* type III (Matsumoto *et al*., 2000), showing 98% identity, corresponding to 15 nucleotide differences in 932 bp. Morphologically the *P.* '*vipa*' strains did not fit the descriptions of *P. irregulare* type III or any other *Pythium* species. *Pythium* '*vipa*' did not produce zoospores. Oogonia were on average about 20 μm and oospores 17 μm in size. Oogonia with projections were never observed. Antheridia were mostly monoclinous and sessile, but diclinous antheridia were also common. The modal numbers of antheridia per oogonium were one or two. A phylogenetic tree was constructed including most of the *Pythium* species described in the literature to be isolated from carrot cavity spots (Fig. 1). The *P.* '*vipa*' isolates were found to correspond to clade F (Lévesque & de Cock, 2004), which included other cavity spot pathogens, *P. sylvaticum* and *P. intermedium*, as well as one isolate of *P. violae* originating from *Viola*.

Species-specific PCR

PCR primers (Table 3) were designed for the species-specific identification of *P. intermedium, P. sulcatum, P. sylvaticum, P. violae* and *P.* '*vipa*', using the most variable regions of the ITS sequences (Fig. 2). The specificity of the PCR assays was tested on a large number of *Pythium* species as well as on fungal isolates from carrots (Tables 1 and 2). All PCR primer pairs showed the predicted species specificity, with a strong PCR band of the expected fragment size in all target species (Table 1) and no signal in the non-target species listed in Table 2. The sensitivities of the PCR assays were determined in tenfold dilutions of DNA (Fig. 3). The *P. intermedium* and *P.* '*vipa*' assays were found to be the most sensitive, with a detection limit of 5 fg. The *P. violae* assay was the least sensitive, with a detection limit of 5 pg. Intermediate sensitivities were found for the *P. sulcatum* and *P. sylvaticum* assays, with detection limits of 0·5 pg.

Detection in soil and carrot tissue

The ability of the PCR assays to detect the cavity spot pathogens in carrot tissue with and without visible symptoms was investigated. All five *Pythium* species could be detected by PCR from carrots with visible cavity spot symptoms. Sometimes more than one species could be identified from the same spot (results not shown). In some samples of carrots without visible symptoms, weak positive signals could be obtained. In five out of eight random samples of symptomless carrots, low levels of *P. intermedium*, *P. sulcatum* and *P.* '*vipa*' were detected (Fig. 4).

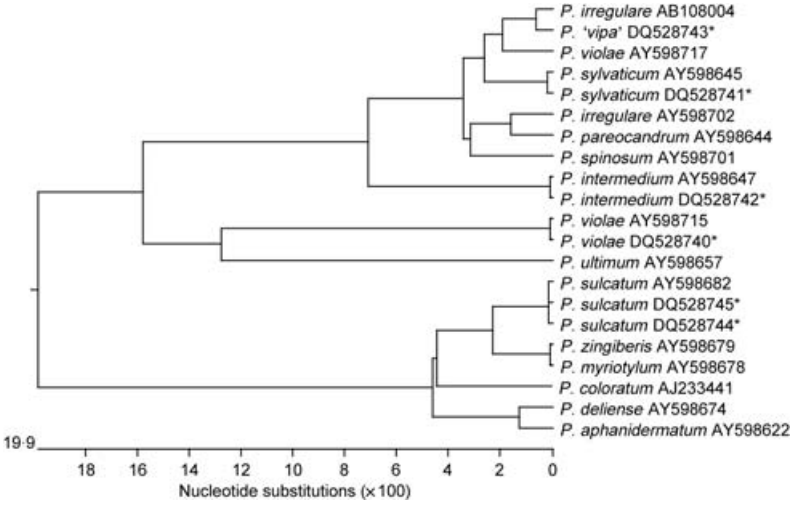

Figure 1 Phylogenetic relationships among carrot-pathogenic and closely related *Pythium* species inferred from the nucleotide sequence of ITS (1 and 2) and the intervening 5·8S gene of nuclear rDNA. For each species and isolate GenBank Accession Nos. are given. Sequences determined as a part of this study are marked by asterisks.

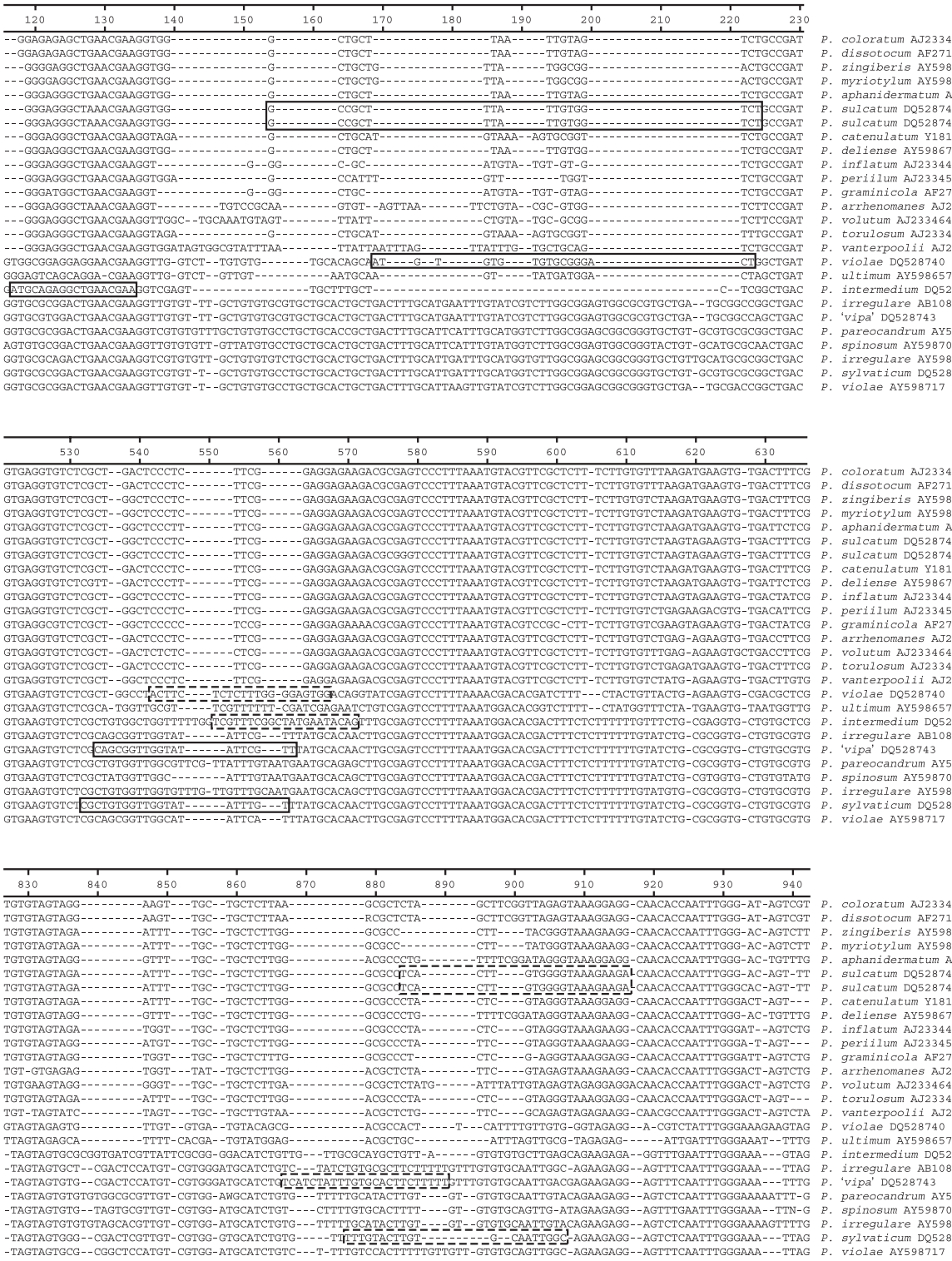

Figure 2 Sequence alignments of the three most variable regions of ribosomal rDNA used for the design of *Pythium* species-specific primers. All primer sequences are boxed. Forward primers are boxed with a solid line and reverse primers are boxed with a dotted line.

In soils known to produce cavity spots on cropped carrots, relatively strong signals were obtained and in several soil samples more than one of the five *Pythium* species could be detected (Fig. 5). In a field where 79·7% of carrots had symptoms of cavity spots at harvest, all five *Pythium* species could be detected in the soil samples by PCR (Table 4), but of *P.* '*vipa*' only minor amounts could be detected. Most frequently found were *P. intermedium*

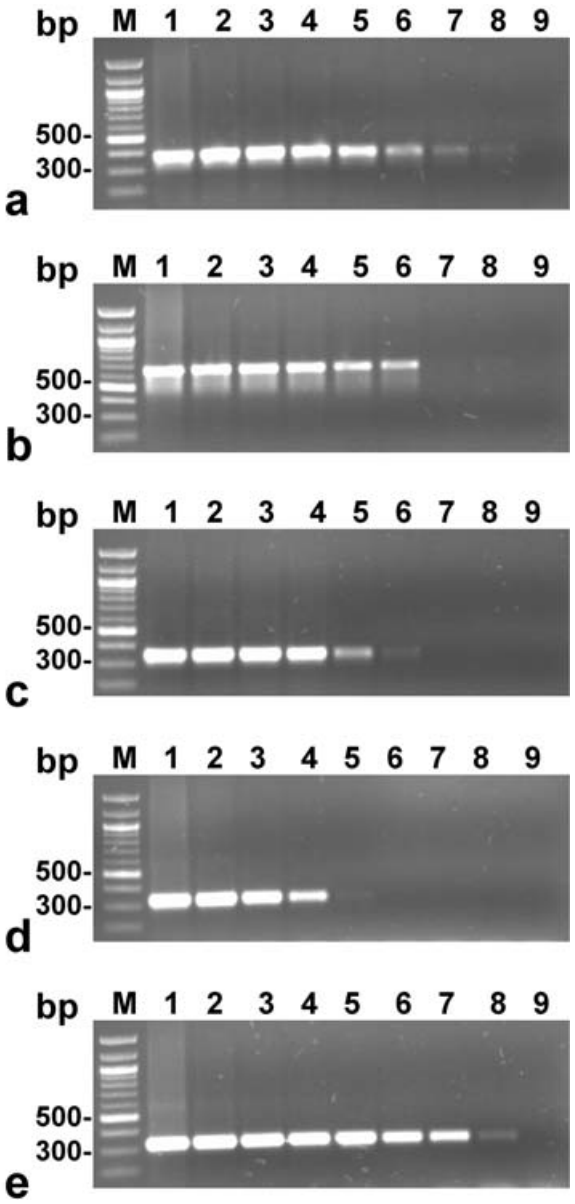

Figure 3 Sensitivities of species-specific PCR tests for *Pythium intermedium* (a), *P. sulcatum* (b), *P. sylvaticum* (c), *P. violae* (d) and *P.* '*vipa*' (e) with the primer pairs PINTERf/r, PSULCf/r, PSYLf/r, PVIOLf/r and P*VIPA*f/r, respectively. Amplification was performed using decreasing amounts of template genomic DNA ranging from 50 ng to 5 fg (lanes 1–8). Lane 9 is a negative control where DNA was replaced by water. A 100-bp ladder was used as the molecular marker (M).

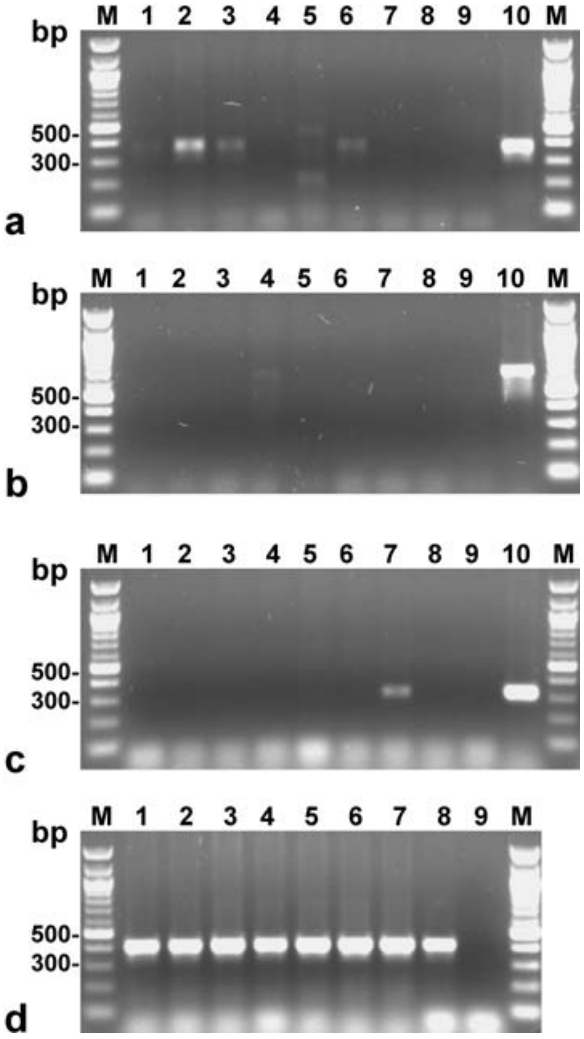

Figure 4 Detection of cavity spot pathogens *Pythium intermedium* (a), *P. sulcatum* (b) and *P.* '*vipa*' (c) in eight random symptomless carrot samples (lanes 1–8) with the primer pairs PINTERf/r, PSULCf/r and P*VIPA*f/r, respectively. A PCR reaction with primer pair ITS3 and ITS4 was used as a control reaction (d). Lane 9 is a negative control where DNA was replaced by water. Pure DNA from the respective species was used as a positive control (lane 10). A 100-bp ladder was used as the molecular marker (M).

and *P. sulcatum*. Less frequently found were *P. violae* and *P. sylvaticum*, although in some samples relatively high levels of these pathogens were observed.

To test the specificity of the PCR assays in such complex samples as soil samples, the DNA sequence was determined directly from the amplification products originating from five independent fields for each of the five primer combinations. For each of the assays one PCR product was cloned and the DNA sequence determined for five random clones. The specificities of the assays were confirmed as the sequences were found to be identical to those of the positive control strains.

Discussion

A PCR assay is presented that allows specific identification of all five *Pythium* species found to be most important for the development of cavity spot in Norway and their specific detection in soil and carrot tissue. DNA sequence analysis also confirmed the specificity of the PCR primers in the detection of the particular *Pythium* species in complex samples such as soil samples. The detection limits of the PCR assays presented here differed among

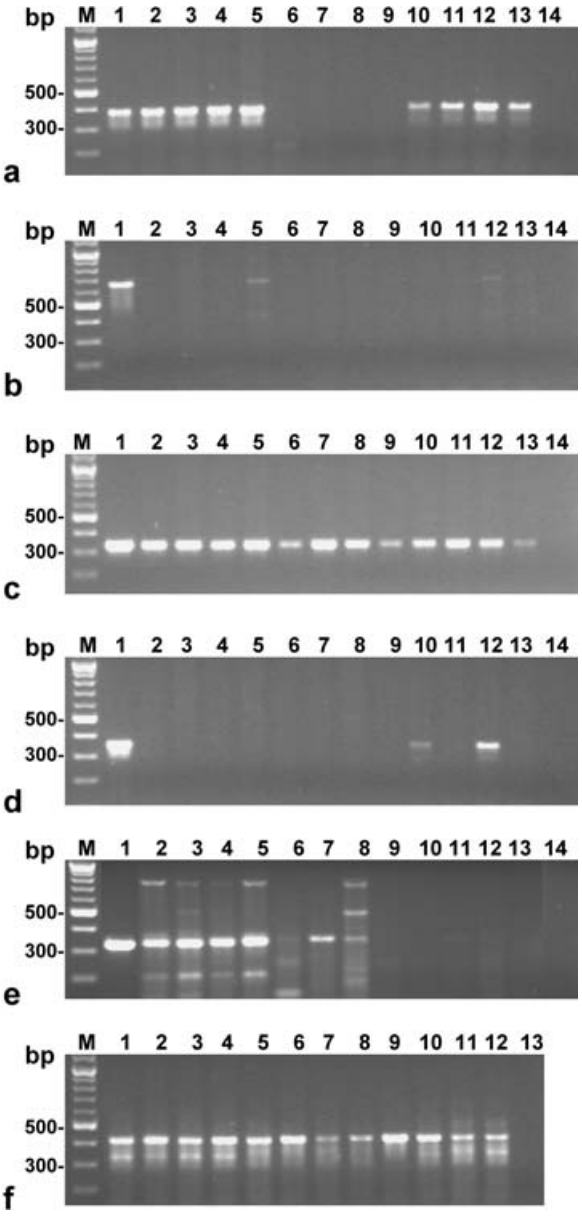

Figure 5 Detection of the cavity spot pathogens *Pythium intermedium* (a), *P. sulcatum* (b), *P. sylvaticum* (c), *P. violae* (d) and *P.* '*vipa*' (e) in 12 samples of soil (lanes 2–13) with the primer pairs PINTERf/r, PSULCf/r, PSYLf/r, PVIOLf/r and P*VIPA*f/r, respectively. All samples represent different soils. Pure DNA was used as a positive control (lane 1). Lane 14 is a negative control where DNA was replaced by water. PCR with primer pair ITS3 and ITS4 was used as a control reaction (f). The 12 soil samples were loaded in lanes 1–12. Lane 13 is the negative control where DNA was replaced by water. A 100-bp ladder was used as the molecular marker (M).

the primer pairs. Most sensitive were the detection of *P. intermedium* and *P.* '*vipa*', where as little as 5 fg DNA resulted in a weak PCR fragment. *Pythium* species have been estimated to have genomes ranging from 18·8 to 41·5 Mb (Kamoun, 2003), equivalent to 0·035–0·077 pg DNA. The detection limits observed for the *P. intermedium* and *P.* '*vipa*' assays would then correspond to the detection

Table 4 Results from PCR analysis of eight soil samples from a carrot field where the carrots were heavily diseased with cavity spot. The geometric mean, median, minimum and maximum values of the species-specific amplification product of the five *Pythium* species analysed, as determined by Quantity One software, are presented as percentages of the positive control sample

	Mean	Median	Minimum	Maximum
P. intermedium	61·4	80·8	0·0	100·0
P. '*vipa*'	0·0	0·0	0·0	0·1
P. sulcatum	38·3	46·8	0·0	79·2
P. sylvaticum	6·2	0·0	0·0	80·7
P. violae	4·4	6·2	0·0	24·5

of less than one genome of the target species in the reaction mixture. This can be explained by the fact that the PCR primers were based on the ITS regions, which exist in multiple copies per genome. Similar sensitivity was observed in other PCR-based diagnostic assays designed from the ribosomal DNA region (e.g. Suhara *et al*., 2005; Zeng *et al*., 2005). The *P. violae* PCR assay was the least sensitive, although also these PCR primers allowed the detection of the pathogen in field soil as well as in carrot tissue. Further studies should be directed to improve the sensitivity of this assay.

The *P.* '*vipa*' isolates probably represent a new *Pythium* species and were found to be the most aggressive *Pythium* species on carrots (Hermansen *et al*., 2007). Similar isolates with ITS sequence most similar to *P. irregulare* type III (Matsumoto *et al*., 2000) were previously described only from sugar beet or from soil cropped with sugar beet in Japan. Several morphological differences, as well as 2% difference in the ITS sequence, indicate that *P.* '*vipa*' and *P. irregulare* type III represent two different but genetically closely related species. For some soil samples amplification with the *P.* '*vipa*'-specific primers resulted in strong PCR bands of the correct fragment length, indicating that the amount of this species in the soil might be relatively high. The fact that such a PCR fragment was also amplified when analysing soil samples from Denmark, Sweden and the UK (results not shown) indicates that *P.* '*vipa*' is not specific to Norway. However, a pure isolate of *P.* '*vipa*' from field soil or carrots from these other countries has not yet been obtained.

The method may also be of value to carrot growers in other geographic regions, since *P. violae*, *P. sulcatum* and *P. sylvaticum* are reported to cause cavity spot in several other countries (reviewed by White, 1986; Allain-Boulé *et al*., 2004a). *Pythium intermedium* was previously detected in carrot cavities in the UK (White, 1988), but unlike the situation in Norway (Hermansen *et al*., 2007), it was considered to be a secondary and not a primary pathogen (Lyons & White, 1992). More investigation is therefore needed to determine the relative importance of this pathogen for the development of cavity spot in other geographic regions. Recently, Suffert & Guibert (2007) performed a thorough study of the ecology of *Pythium* species in relation to the epidemiology of carrot cavity

spot in France. They found that in addition to *P. violae* and *P. sulcatum*, which were characterized as the major pathogens, *P. intermedium* was among the most important 'minor' pathogenic species, inducing a maximum of 25% of the symptoms. Although cavity spot infects and develops in the growing season, symptoms have been found to increase during long-term storage, either under straw on the field or indoors in cold-storage rooms (Lyngstad, 1991; Phelps *et al*., 1991). Knowledge of the presence and abundance of cavity spot pathogens on carrots might enable the carrot grower to select the best lots for long-term storage and direct the most infected carrots towards immediate consumption. Furthermore, a pre-drilling PCR test of field soil might help to select the least infected fields for carrot production and thereby reduce the risk of disease development. Depending on the time of sampling the assays might potentially also be used to predict the need for fungicide treatment and consequently reduce the amount of pesticides used in carrot production. The utilization of these diagnostic assays on field soil and carrot tissue might therefore contribute to a future reduction of the loss of carrot crops caused by cavity spot. The PCR analysis performed in this study is not a quantitative analysis. Characterizing the PCR products as described for the soil samples can at best be characterized as semi-quantitative. The results indicate, however, that the PCR primers described here might have future potential in the analysis of soil samples for the presence of cavity spot pathogens. Investigations are now in progress to establish the best sampling procedure and time and to study the correlation between the results from the molecular diagnostic tests and the development of cavity spot in carrots.

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